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Award Number: W81XWH-05-1-0241

TITLE: Extranuclear Signaling Effects Mediated by the Estrogen Receptor

PRINCIPAL INVESTIGATOR: Erin O'Neill, B.S.
Geoffrey Greene

CONTRACTING ORGANIZATION: University of Chicago
Chicago, Illinois 60637

REPORT DATE: March 2006

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
March 2006	Annual Summary	15 Feb 05 – 14 Feb 06

Extranuclear Signaling Effects Mediated by the Estrogen Receptor

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-05-1-0241

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Erin O'Neill, B.S.
Geoffrey Greene

E-mail: eionson@uchicago.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Chicago
Chicago, Illinois 60637

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Recent evidence has made it clear that ER-mediated extranuclear signaling is involved in the growth and survival of ER-expressing cells and tissues, including the mammary gland. Specifically, we are interested in examining the ability of ER action to modulate MAPK signaling as well as other key signaling molecules, and our main goals with this research are to: 1) better define the mechanism responsible for the observed cross-talk, 2) investigate the observed signaling in an animal model, 3) determine and compare the target genes that are regulated by ER rapid signaling versus classical ER transactivation, and 4) examine the subsequent cellular and biological responses to rapid 17β-estradiol (E2) action. Previously, we confirmed that E2 and other ER-specific ligands can rapidly phosphorylate and activate Erk-1 and -2 in the breast cancer cell line, MCF-7, an effect that is blocked by the potent ER antagonist, ICI 182, 780. We have also provided preliminary evidence that demonstrated that E2 administration to ovariectomized immature rats can induce Erk-1 and -2 phosphorylation in the uterine horn. We show here our continued investigation of ER-mediated Erk-1 and -2 activation in vivo, and that E2 administration can, in fact, result in a significant increase of Erk-1 and -2 phosphorylation over saline control in both the uterine horn and brain. In each case, E2-induced Erk-1 and -2 activation is at least partially decreased by the co-administration of raloxifene, a selective estrogen receptor modulator (SERM). Additionally, we have identified CaMKII as an important player in ER-mediated Erk-1 and -2 activation. In cultured neuronal cells, the inhibition of CaMKII with KN-62 blocks E2-induced Erk-1 and -2 phosphorylation. Interestingly, as little as 15 minutes of E2 administration to ovariectomized rats results in a significant increase in CaMKII autophosphorylation and thus, its activation in the brain. We are continuing to examine this effect in the uterine horn and future experiments will include harvesting the mammary gland to investigate E2-induced Erk-1 and -2 and CamKII activation in that tissue. The completion of this work will result in an improved understanding of ER signaling outside of its classical genomic role, and has the potential to ultimately lead to the discovery of novel therapeutic targets for ER-positive breast cancer.

15. SUBJECT TERMS
Estrogen Receptor, Rapid Extranuclear Signaling, Erk-1 and -2, CaMKII

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
					USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
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Introduction:

Recent evidence has made it clear that ER-mediated extranuclear signaling is involved in the growth and survival of ER-expressing cells and tissues, including the mammary gland (1, 2). We have pursued this research in order to examine the ability of ER action to modulate MAPK signaling as well as other key signaling molecules. We will better define the mechanism responsible for the observed cross-talk and analyze the signaling in an animal model. In order to gain insight into the downstream effects of ER-mediated extranuclear signaling, we will determine and compare the target genes that are regulated by ER rapid signaling versus classical ER transactivation, and examine the subsequent cellular and biological responses. This work will result in an improved understanding of ER signaling outside of its classical genomic role, and has the potential to ultimately lead to the discovery of novel therapeutic targets for ER-positive breast cancer.

Body:

Previously, we confirmed that 17β-estradiol (E2) can rapidly phosphorylate and activate Erk-1 and -2 in the breast cancer cell line, MCF-7. A variety of other ligands including diethylstilbestrol (DES), propyl pyrazole triol (PPT), and 4-estren-3α-17β-diol (Estren) can also stimulate the rapid activation of Erk-1 and -2, an event that is effectively inhibited by the potent ER antagonist, ICI 182, 780 (ICI). Additionally, we performed a pilot animal experiment with ovariectomized immature rats where we demonstrated that E2 can induce Erk-1 and -2 phosphorylation in the rat uterine horn when injected intraperitoneally, an effect that is partially blocked by simultaneous ICI injection. This experiment, however, was quite preliminary; only one animal was examined at each time point for each ligand injected. Clearly, we needed to provide more significant evidence to confirm our initial findings so we continued to examine ER-mediated extranuclear signaling *in vivo* using the ovariectomized immature rat as a model.

Our general experimental protocol is as follows: 3-6 rats per treatment/time group were injected either intraperitioneally or subcutaneously with ligand, and at the indicated time points the uterine horns and brain were harvested for further analysis. More tissues, including the mammary gland, will be harvested in future experiments. In Figure 1 of the appendix, we show that intraperitoneal injection of E2 results in a significant increase of Erk-1 and -2 phosphorylation over saline control in the uterine horn by 2 hours as evidenced by Western blot analysis. This peak of activation decreases back to baseline levels by 24 hours. Next, we wanted to confirm that we could reproduce this effect when ligand was injected subcutaneously. Figure 2 shows that 2 hours of E2 administered subcutaneously can, in fact, induce a significant increase in Erk-1 and -2 activation over saline control in the uterine horn. It is apparent, however that it resulted in a less dramatic effect when compared to the increase produced by intraperitoneal injection of E2. Additionally, we found that raloxifene injected subcutaneously 2 hours prior to E2 can partially block the E2-induced Erk-1 and -2 activity. We are currently testing to determine if the 2 hour pre-treatment with raloxifene is the optimum time point required for blocking this signaling event in the uterine horn.

A second tissue we are interested in examining is the brain as this is a major tissue that is affected by estrogen action. Figure 3 shows that intraperitoneally administered E2 induces a rapid increase in Erk-1 and -2 activation over saline control in whole brain extract by 30 minutes, an effect that peaks at 2 hours and returns to baseline levels by 24 hours post-injection. Interestingly, ICI is unable to block this response when administered intraperitoneally 2 hours prior to the E2 injection. Raloxifene, however, is able to almost completely inhibit the E2-induced Erk-1 and -2 phosphorylation when injected intraperitoneally 2 hours before E2 administration. All of the tissues used in these experiments have been frozen and stored for future use to determine the phosphorylation status of pRb and cyclin D1 protein expression as proposed in the statement of work, task 1. This information will help us to better understand the downstream cellular response to the ER-mediated kinase activation.

In order to examine the key signaling molecules involved in the E2-induced Erk-1 and -2 activation, both MCF-7 and NLT cells were used. We are continuing are studies with MCF-7 cells, unfortunately, we have obtained inconsistent results using this cell line thus far so there is no conclusive data to report at this time. NLT is a neuronal cell line consisting of gonadotropin releasing hormone (GnRH) neurons and we utilized these cells as they were readily available to us, and because we observed a dramatic increase in Erk-1 and -2 activation by E2 in rat brain extract. In Figure 4a we show that E2 and PPT can induce Erk-1 and -2 phosphorylation within 10 minutes of treatment, an effect that is blocked by ICI treatment. Interestingly, pre-treatment with KN-62, a Ca^{2+}/CaM-dependent kinase II (CaMKII)-specific inhibitor, is significantly effective at blocking E2-induced Erk-1 and -1 activation. This result gives us our first glimpse into a novel mechanism by which ER can signal to Erk-1 and -2. Importantly, we also show that E2 can rapidly induce the autophosphorylation and activation of CaMKII itself within 10 minutes as shown in Figure 4b. We are interested in CaMKII as a potential partner in ER extranuclear signaling as it is a ubiquitous kinase that a former graduate student in the lab previously reported to interact with ER in breast cancer cells. She also demonstrated that CaMKII can phosphorylate ER in the ligand-binding domain, a modification which enhances the receptor's ability to function as a transcription factor in breast cancer cells. An antibody microarray will be used in the future to fully investigate and understand the complexities of ER-mediated rapid signaling. Finally, Figure 5 shows that E2 can induce the autophosphorylation of CaMKII in the brain of ovariectomized rats when injected intraperitoneally. The increase in autophosphorylation begins as soon as 15 minutes post-injection, peaks at 1 hour, and returns almost to baseline by 12 hours. ER clearly plays a role in the autophosphorylation and thus activation of CaMKII *in vivo* and it will be interesting to determine if KN-62 injection will block E2-induced Erk-1 and -2 activation in the ovariectomized rat.

The remaining tasks in the statement of work will be addressed and completed in future experiments.

Key Research Accomplishments:

- Provided evidence that E2 can induce Erk-1 and -2 phosphorylation in the rat uterine horn and brain when injected intraperitoneally or subcutaneously.
- Raloxifene co-administration can partially block E2-stimulated Erk-1 and -2 activation in the rat uterine horn and brain. ICI is unable to inhibit E2-induced Erk activation in the brain.
- CaMKII appears to be involved in ER-mediated Erk signaling.

Reportable Outcomes:

Abstracts to be presented:

O'Neill, E., Blewett, A., Loria, P., Greene, G.
Keystone Symposia, Nuclear Receptors: Steroid Sisters Meeting
Fairmont Banff Springs. Banff, Alberta, Canada – March 2006

O'Neill, E., Blewett, A., Loria, P., Greene, G.
Biomedical Sciences Retreat, University of Chicago
Grand Geneva Resort and Spa. Lake Geneva, WI - May 2006

Conclusion:

Overall, the data presented here begins to address the question of how ER modulates the Erk-1 and -2 signaling cascade outside of the nucleus. We have provided evidence that this signaling can occur *in vivo* using the ovariectomized immature rat as a model. We have also provided data that implicates CaMKII as a signaling partner in ER-mediated Erk-1 and -2 activation. Alterations to the original statement of work are required in order to more completely address our original hypothesis. We need to more thoroughly investigate the relationship between ER and CaMKII signaling, especially since it was previously shown in our lab that they can interact in a functionally relevant manner in breast cancer cells: CaMKII phosphorylates ER which then enhances its ability as a transcription factor. It is incredibly interesting that ER can, in turn, stimulate the autophosphorylation of CaMKII and we still need to explore and understand the biological significance of this event. The antibody microarray experiments proposed in Task 1 will not only examine molecules essential for Erk-1 and -2 signaling, but those that are also involved in CaMKII action. CaMKII plays a variety of roles in a wide range of cellular processes, and subsequently, it has a large number of substrates; we want to characterize the role, if any, of these proteins in ER extranuclear signaling (3, 4). Additionally, it is important to follow-up on the data that we have initially obtained in the NLT GnRH cell line as the central nervous system (CNS) is a major site for estrogen action and the mechanisms are still not fully understood (5,6).

The outcome of this completed work has several implications. First, it will result in an improved understanding of ER signaling outside of its classical genomic role. Second, if we continue to examine ER-mediated extranuclear signaling in neuronal cells and the brain, it has the promise to develop advancements in hormone replacement therapies as it will result in a better understanding of how estrogen functions within the central nervous system. Finally, and perhaps most notably, this work has the potential to ultimately lead to the discovery of novel therapeutic targets for ER-positive breast cancer.

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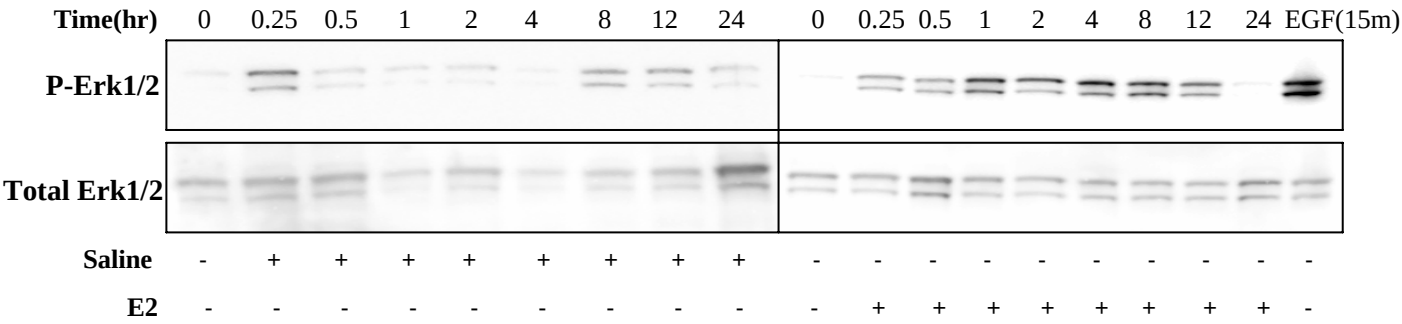
Appendix 1: Supporting Data

a.



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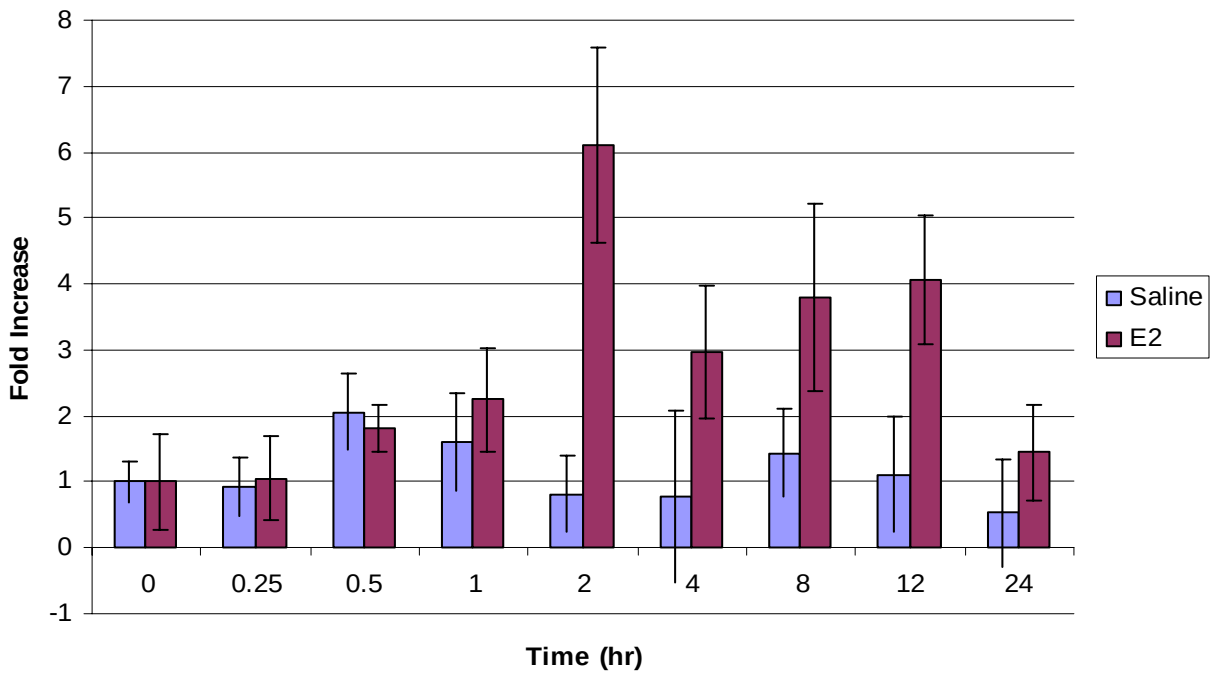


Figure 1. Estrogen stimulates Erk-1 and -2 activation in rat uterine horns. a) ovariectomized female rats (21 days old) were intraperitoneally injected with either saline control or E2(0.1ug) or EGF (0.1ug) for the time period indicated. The uterine horns were then removed, homogenized, and western blot analysis was performed to detect Erk-1 and -2 phosphorylation relative to total Erk-1 and -2 expression. Representative of 6 replicates. b) Graphic representation of all 6 replicates.

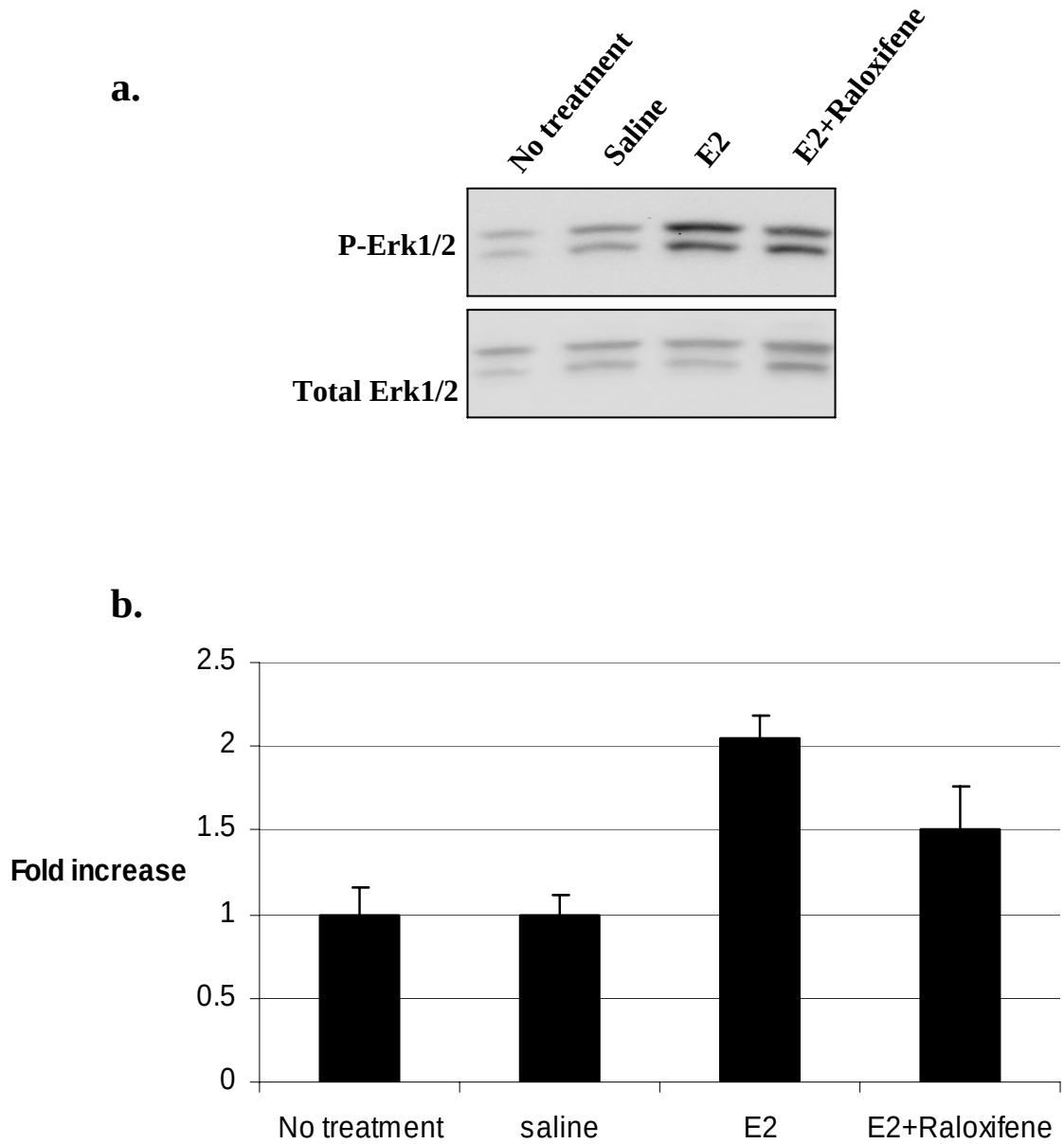
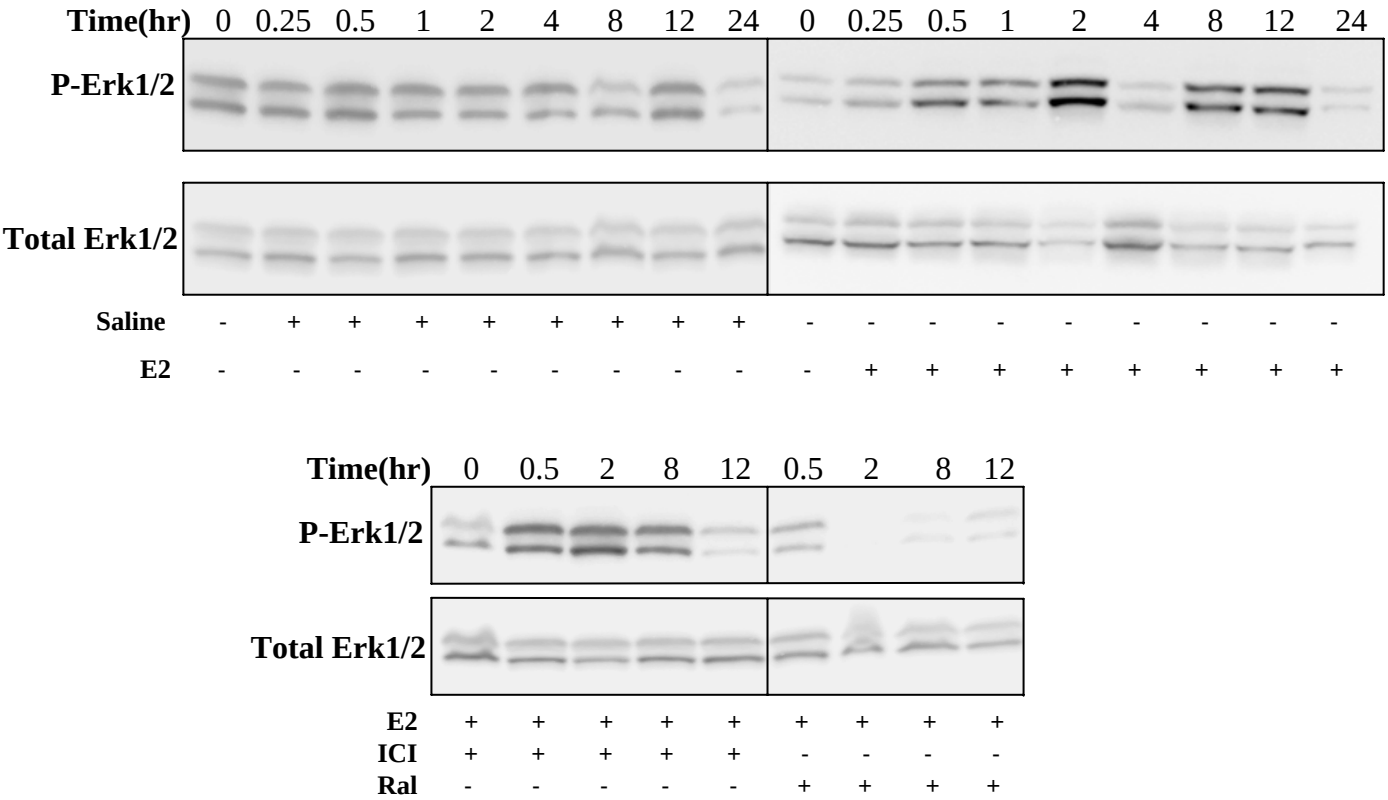


Figure 2. Raloxifene can partially block E2-stimulated Erk-1 and -2 activation in rat uterine horns. a) ovariectomized female rats (21 days old) were injected subcutaneously with either saline control, E2(0.1ug), or E2 (0.1ug) + raloxifene (10ug) for 2 hours. Raloxifene was injected 2 hours prior to E2 administration. The uterine horns were removed, homogenized, and western blot analysis was performed to detect Erk-1 and -2 phosphorylation relative to total Erk-1 and -2 expression. Representative of 3 replicates. b) Graphic representation of all 3 replicates.

a.



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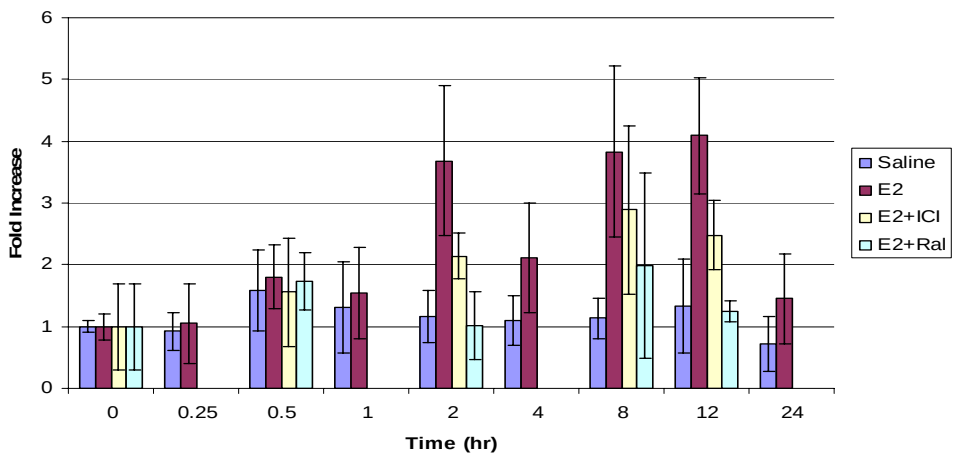
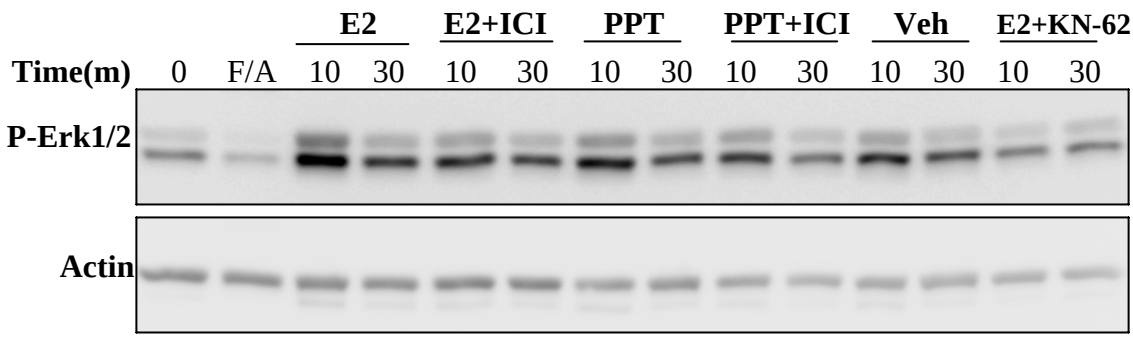


Figure 3. E2 stimulates Erk-1 and -2 activation in rat brain. a) ovariectomized female rats (21 days old) were intraperitoneally injected with either saline control or E2(0.1ug) as shown in the top panel or with either E2(0.1ug)+ICI(10ug) or E2(0.1ug)+raloxifene(10ug) as shown in the bottom panel for the time periods indicated. ICI and raloxifene were injected 2 hours prior to E2 administration. The brain was then removed, homogenized, and western blot analysis was performed to detect Erk-1 and -2 phosphorylation relative to total Erk-1 and -2 expression. The top panel is representative of 6 experiments and the bottom panel is representative of 3 experiments. b) Graphic representation of all the indicated replicates.

a.



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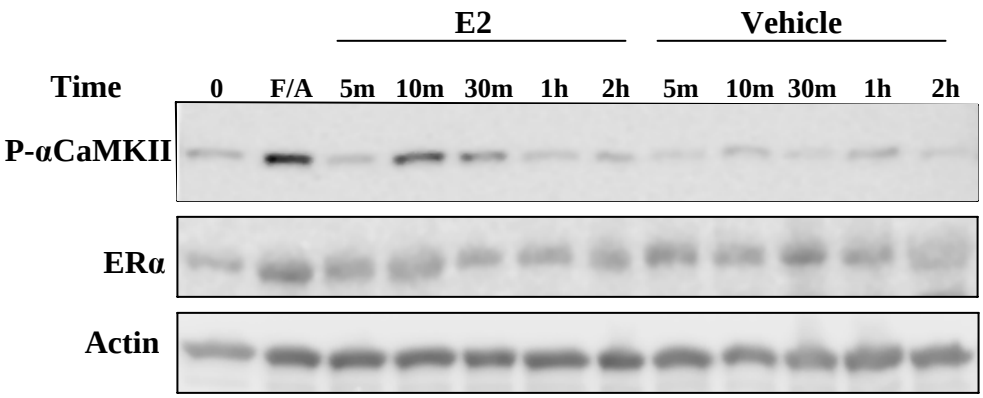
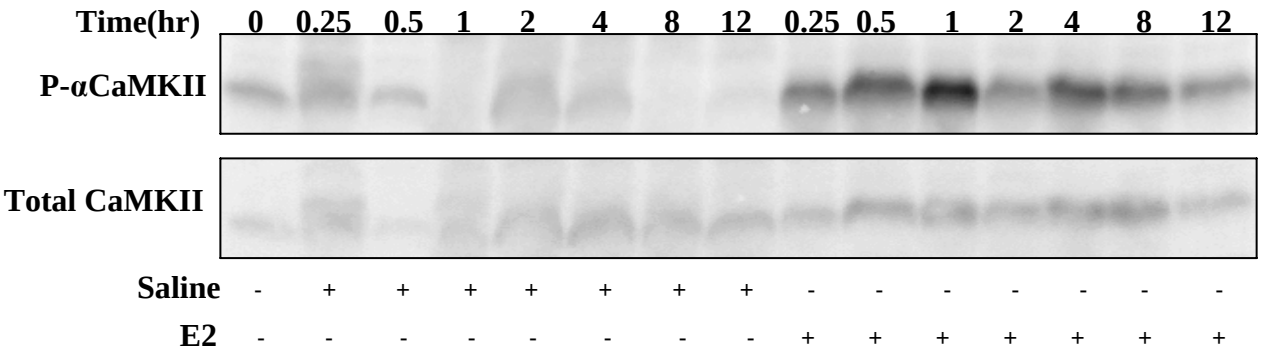


Figure 4. CaMKII is involved in ER-mediated Erk-1 and -2 activation. a) NLT cells were treated with either forskolin/A23187 (F/A), E2 (10nM), E2+ICI(1uM), PPT (10nM), PPT+ICI(1uM), vehicle, or E2+KN62(10uM) for the indicated time points. The cells were lysed and western blot analysis was performed on the resulting lysate to detect phosphorylated Erk-1 and -2 levels relative to actin. Representative of 3 separate experiments. b) NLT cells were treated with either E2(10uM) or vehicle for the indicated time, lysed, and then used in western blot analysis to detect phosphorylated CaMKII and ER levels relative to actin. Representative of 3 separate experiments.

a.



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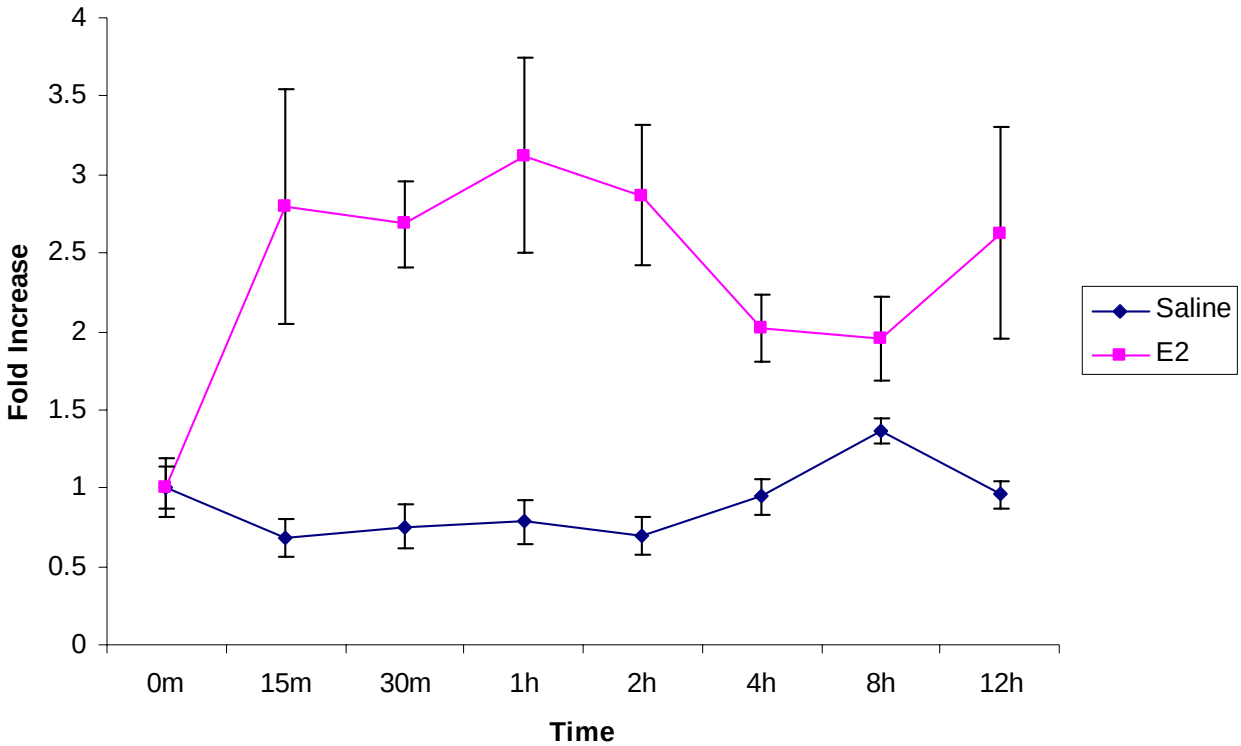

Figure 5. E2 stimulates αCaMKII autophosphorylation in rat brain. a) ovariectomized female rats were injected intraperitoneally with saline or E_2 (0.1ug) and the brain was extracted at the indicated time points. The tissue was homogenized and autophosphorylated (T286) αCaMKII was determined relative to total αCaMKII levels by western blotting. Representative of 6 replicates. b) Graphic representation of all 6 replicates.